3-26-2018

Cellulose Microfibril Structure: Inspirations from Plant Diversity

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Citation/Publisher Attribution

Roberts, A.W. Cellulose Microfibril structure: Inspirations from Plant Diversity (2018) *IOP Conference Series: Earth and Environmental Science*, 130(1), art. no. 012001. DOI: 10.1088/1755-1315/130/1/012001

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Abstract. Cellulose microfibrils are synthesized at the plasma membrane by cellulose synthase catalytic subunits that associate to form cellulose synthesis complexes. Variation in the organization of these complexes underlies the variation in cellulose microfibril structure among diverse organisms. However, little is known about how the catalytic subunits interact to form complexes with different morphologies. We are using an evolutionary approach to investigate the roles of different catalytic subunit isoforms in organisms that have rosette-type cellulose synthesis complexes.

1. Diversity in cellulose microfibril structure and biosynthesis

Cellulose is an important component of many commercial products and a carbon-neutral energy source. It is produced by diverse organisms in the form of microfibrils, which consist of bundles of 1,4-β-glucan chains stabilized by inter-chain hydrogen bonds. These microfibrils are synthesized by cellulose synthase catalytic subunits that associate in the plasma membrane to form Cellulose Synthesis Complexes (CSC). The number and arrangement of catalytic subunits varies among different taxa, and this variation in CSC organization has been correlated with variation in cellulose microfibril structure [1]. For example, linear CSCs in bacteria [2], brown algae [3], dinoflagellates [4], and red algae [5, 6] produce ribbon-like cellulose microfibrils. In some chlorophycean green algae, large CSCs produce microfibrils containing more than 100 glucan chains [7]. The CSCs of land plant and their close charophycean green algal relative are hexagonal structures known as rosettes ([8], which contain 18 catalytic subunits and therefore produce cellulose microfibrils consisting of 18 glucan chains [9]. These rosette CSCs are composed of specialized cellulose synthases designated CESAs [10, 11] and it has been proposed that domains unique to CESAs participate in CSC assembly [12]. The CESAs diversified early in the evolution of the seed plant lineage to form a medium-sized family whose members are functionally-specialized [13]. Although nearly all commercial cellulose is derived from organisms that have CESAs and rosette CSCs, we know little about the evolutionary origin of CESA proteins or the selective pressures that drove their functional specialization.

2. CESAs functional specialization in Arabidopsis

The specialized functions of the ten members of the Arabidopsis CESA family have been well characterized. First, it has been shown that different CESAs are responsible for primary and secondary cell wall deposition. Specifically, the cellulose microfibrils in secondary cell walls are synthesized by AtCESA4, -7 and -8 [14] and those in in the primary cell walls are synthesized by AtCESA1, -3, and members of the 6-like group [15, 16]. However, promoter swap experiment have shown that at least some primary AtCESAs can substitute for secondary AtCESAs and vice versa, i.e. *AtCESA3pro::AtCESA7* partially rescues *atcesa3* and *AtCESA8pro::AtCESA1* partially rescues *atcesa8* [17]. This is consistent with a role for sub-functionalization in the evolution of seed plant *CESA*s. A role for neo-functionalization is reflected in the shared phenotype of *atcesa4*, *atcesa7,* and *atcesa8* null mutants [18] and the inability to complement these mutants by expressing one of the other secondary *AtCESA*s with the promoter for the missing isoform [19]. This, combined with similar studies of the primary AtCESAs [15, 16], suggests a requirement for three functionally distinct, non-interchangeable CESA isoforms for both primary and secondary cell wall biosynthesis. In addition, there is some functional redundancy among the 6-like members of the *AtCESA* family, *AtCESA2*, *-5*, *-6* and *-9* [16].

3. *Physcomitrella patens* as a model for investigating CESA and CSC evolution

Physcomitrella patens represents the mosses, a lineage of non-vascular plants that diverged from the seed plant lineage over 440 million years ago [20]. The life cycle of *P. patens* [21] includes a haploid phase consisting of a system of protonemal filaments and leafy gametophores (Fig. 1), and a diploid phase that consists of short-stalked sporangia. Although true vascular tissue is lacking, the leaf midribs of *P. patens* contain supportive stereid cells with thick secondary cell walls and thin-walled hydroids that function in transport [22]. The ability to make targeted changes in the *P. patens* genome by homologous recombination [23] along with a sequenced genome [24, 25] has led to the extensive use of this organism to study the evolution of developmental mechanisms in plants [26, 27].

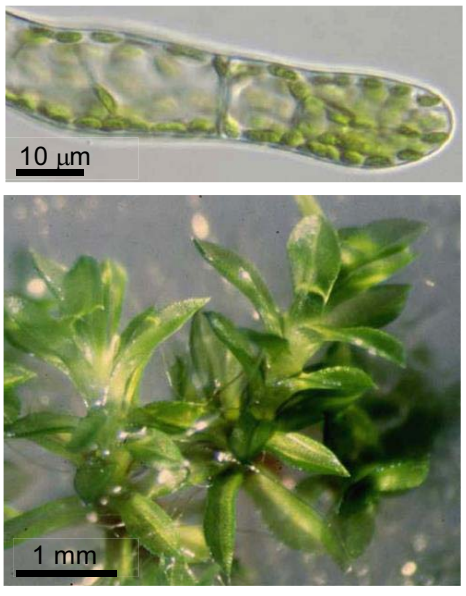

Figure 1. Protonemal filament (top) and leafy gametophores (bottom) of *P. patens*.

Like other land plants, *P. patens* has rosette CSCs [9, 28]. The genome of *P. patens* contains seven *CESA* genes and phylogenetic analysis has shown that the moss and seed plant *CESA* families evolved independently from a single common ancestral gene [19, 29-31]. Thus, the diversification and functional specialization of *CESA*s followed independent paths in the two lineages [28]. We have investigated *CESA* functional specialization in *P. patens* using a targeted gene knockout approach in an effort to better understand the selective pressures that drove the evolution of *CESA* families.

4. CESA functional specialization in *P. patens*

Knock out of individual *PpCESA*s revealed that *PpCESA5* is the only family member with an obvious single-knockout morphological phenotype. The *ppcesa5*KO lines are distinguished by their inability to produce leafy gametophores (Fig. 2). Gametophore buds develop normally through the 4-cell stage, but these cells expand and divide abnormally, producing irregular cell clumps instead of an organized leaf-generating meristem [32]. The defects in cell division and cell expansion are consistent with a role for PpCESA5 in biosynthesis of the primary cell wall.

Figure 2. Phenotype of *ppcesa5*KO with irregular clumps of tissue (arrows, left), in place of wild type leafy gametophores (right).

The production of double knockouts revealed phenotypes for *ppcesa3/8*KO, *ppcesa6/7*KO and *ppcesa4/10*KO. In *ppcesa3/8*KO and *ppcesa6/7*KO lines, the leaf midribs showed reduced birefringence and staining with the cellulose binding dye Pontamine Falst Scarlet 4B (S4B; Fig. 3), consistent with cellulose deficiency [33]. Labeling of sections with the carbohydrate binding module 3A indicated that the secondary cell walls of the stereid cells in the leaf midribs were cellulose deficient [33]. Quantitative analysis of S4B fluorescence revealed a slight deficiency in midrib cellulose deposition in *ppcesa8*KO. However, the *ppcesa*3KOs were indistinguishable from the wild type, indicating that *PpCESA3* and *PpCESA8* are partially redundant. The *ppcesa3/8*KO phenotype could not be rescued by expressing *PpCESA6, PpCESA7, PpCESA4* or *PpCESA10* under control of the *PpCESA8* promoter, indicating that the evolutionary history of the PpCESA family included neofunctionalization. However, we were able to rescue the *ppcesa3/8*KO by expressing *PpCESA5* under control of the *PpCESA8* promoter. This indicates that evolution of the PpCESA family also involved subfunctionalization. The *ppcesa4/10*KO lines have defects in protonemal growth (unpublished) and are currently being investigated.

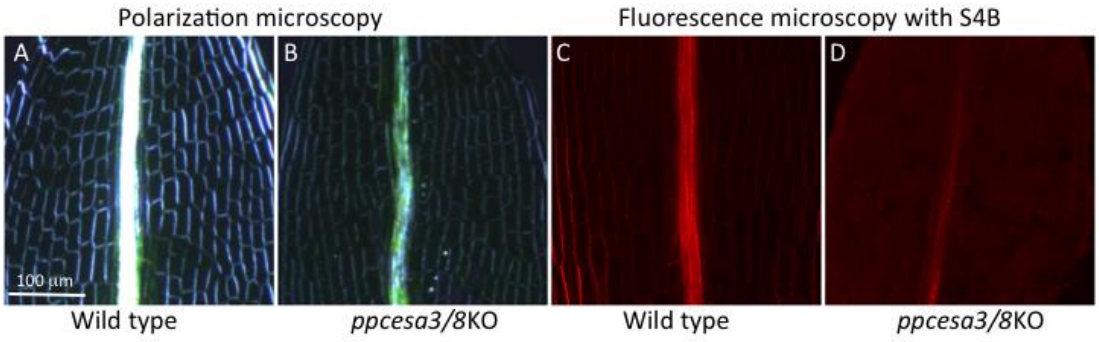

Figure 3. Polarization and fluorescence microscopy of leaves from wild type and *ppcesa3/8*KO *P. patens* showing cellulose deficiency in midribs.

5. Conclusion

Phylogenetic analysis has confirmed that the *CESA* families of seed plants and mosses diversified independently from a single common ancestor [19, 29-31]. However, there are similarities in functional specialization of the *PpCESA* and *AtCESA* families that are attributable to convergent evolution. First, the CESAs that deposit secondary cell walls (*PpCESA3, -6* and *-7*) and primary cell walls (*PpCESA5*) are functionally differentiated [32, 33]. Second, subfunctionalization has occurred as indicated by the ability of *PpCESA5*, which is normally functions in primary cell wall deposition, to rescue the *ppcesa3/8*KO secondary cell wall phenotype when expressed with the *PpCESA8* promoter. Third, neofunctionalization is indicated by the shared phenotype of *ppcesa3/8*KO *and ppcesa6/7*KO and the inability of *PpCESA8pro*::*PpCESA7* to complement the *ppcesa3/8*KO phenotype. We are currently investigating whether the secondary PpCESAs physically interact to form a CSC. Finally, there is some functional redundancy in the PpCESA family since a strong secondary cell wall phenotype is observed only when *PpCESA3* and *-8* or *PpCESA6* and *-7* are knocked out together [33].

Acknowledgment

Work reviewed here was supported by the National Science Foundation under Award IOS-1257047 and the Center for LignoCellulose Structure and Formation, an Energy Frontier Research Center funded by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Award DE-SC0001090.

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